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Report

Drosophila piwi Mutants Exhibit Germline Stem Cell Tumors that Are Sustained by Elevated Dpp Signaling

Zhigang Jin,[1] Alex S. Flynt,[1] and Eric C. Lai[1,*]
[1]Department of Developmental Biology, Sloan-Kettering Institute, 1275 York Avenue, Box 252, New York, NY 10065, USA

Summary

***Drosophila* Piwi is the founding member of a gonadal clade of Argonaute proteins that serve as silencing effectors for ~26–32 nt Piwi-interacting RNAs (piRNAs) [1], and *piwi* mutants exhibit dramatically rudimentary ovaries [2]. It was proposed that somatic Piwi maintains germline stem cells (GSCs) by promoting Dpp signaling, presumably via cap cells that form the somatic niche for GSCs [3–5]. However, we unexpectedly observed that *piwi* mutants exhibit high-frequency GSC-like tumors that persist throughout adult life. Multiple readouts demonstrated hyperactive Dpp signaling in *piwi* mutants, including the failure to express the germline differentiation factor *bag-of-marbles* (*bam*), and restoration of *bam* expression relieved *piwi* GSC-like tumors. Tissue-specific rescue and knockdown experiments indicate that Piwi is not required in cap cells, the source of niche Dpp, but instead is required in gonadal intermingled cells (ICs, the progenitor cells of escort cells). Adult-specific knockdown of *dpp* in escort cells substantially rescued *piwi* tumors, demonstrating that they are driven by excess Dpp signaling. However, the temporal requirement for *piwi* to restrict GSC numbers was much earlier, during the wandering third-instar larval stage. Indeed, *piwi* mutant larval gonads exhibited defective morphology and loss of Bam. Our data indicate that loss of Piwi causes defects in ICs and escort cells, leading to ectopic Dpp signaling and consequent blockage of GSC differentiation.**

Results and Discussion

Drosophila ovaries are composed of ~20 ovarioles, each consisting of a germarium and a string of maturing egg chambers. The germarium tip contains two or three germline stem cells (GSCs), identified by their direct contact with cap cells (CCs) and the presence of ball-shaped spectrosomes stained by Hu-li-tai-shao (Hts); differentiating germline cells maintain Hts expression in branched fusome structures. Ovarian GSCs receive a localized Dpp signal emanating from neighboring somatic niche cells, resulting in phosphorylation of the transcription factor Mothers against *dpp* (pMad), which in turn represses expression of the differentiation factor *bag-of-marbles* (*bam*) [6–8]. Asymmetric GSC division retains one daughter in the niche; this cell maintains Dpp pathway activity and GSC identity. The other GSC daughter loses Dpp signaling and consequently activates Bam, which promotes its differentiation as a cystoblast [9].

piwi mutants exhibit small ovaries, and their ovarioles generate few eggs; initial studies concluded that Piwi was essential to maintain ovarian GSCs [2]. It was later determined that somatic expression of Piwi rescues *piwi* mutant germline defects, leading to a model in which Piwi positively regulates Dpp signaling, presumably in CCs that form the GSC niche [3–5, 10]. We examined this using previously studied *piwi* alleles as well as a deletion allele of the neighboring loci *scar* and *piwi* known as *[Δ37]* [11], which truncates the Piwi protein at residue 745 (E. Schejter, personal communication). We confirmed that *piwi[Δ37]* homozygotes rescued for *scar* expression lack Piwi immunoreactivity in ovaries, as is the case for other *piwi* transheterozygous combinations (see Figures S1A–S1D available online).

The rudimentary size and disorganization of *piwi* ovaries makes them challenging to analyze, and we were also concerned about the potential loss of agametic structures during dissection. We therefore utilized the conventional approach of teasing ovaries apart and examined whole undissected ovaries. The former technique yields flatter mounts of separated ovariole structures for optimal imaging, whereas the latter method is more cumbersome to interpret, as ovarioles are more distributed through the z axis of the tissue, but it ensures that no material was lost during preparation. We obtained similar results with both methods.

Based on previous reports [3–5], we expected to see a substantial population of germaria lacking GSCs in newly born *piwi[1/Δ37]* ovaries. Careful examination revealed several classes of structures. We did indeed identify germaria lacking GSCs, but these were rare. We classified these on the basis of a stack of disc-shaped terminal filament (TF) cell nuclei adjacent to Tj+ CCs, followed by an identifiable germarium structure containing Tj+ escort cells (ECs) but lacking GSCs (Figure 1A). These structures sometimes included an egg chamber, but often they did not. Much more common, however, were TF/CC structures that not only lacked associated germline cells but also lacked ECs (Figure 1B). Although such "orphan" TF/CC clusters lack GSCs, their absence of an intact germarium raised the possibility that they never recruited germline cells during niche formation (as opposed to subsequently losing their GSCs). At present, we cannot distinguish these scenarios, but because "GSC loss" and "orphan TF/CC" classes represented 5.3% and 25.2% of structures, respectively (n = 246), they collectively represent a minority of germaria.

Surprisingly, the strong majority of germaria (69.5%; Figures 1C and S1E) exhibited extra spectrosomes indicative of GSC tumors. We confirmed similar results in newly born *piwi[1/2]* mutants (n = 245; quantified in Figure S1E). We further verified the GSC tumor phenotype in multiple heteroallelic combinations of *piwi[1]*, *piwi[2]*, *piwi[pz]*, and *piwi[Δ37]*, and this phenotype was rescued by the *myc-piwi* transgene (Figures 1D–1G and S1F–S1J). Moreover, *piwi[Δ37]* homozygotes rescued for Scar expression also exhibited ectopic GSC-like cells (Figure S1H).

We continued to observe highly supernumerary spectrosome-containing germline cells at 30 days (Figures 1I and S1K), indicating that this was not a transient phenomenon. At this point, *piwi* mutant ovaries exhibited considerable degeneration and ectopic cell death (Figures S1M and S1N).

*Correspondence: laie@mskcc.org

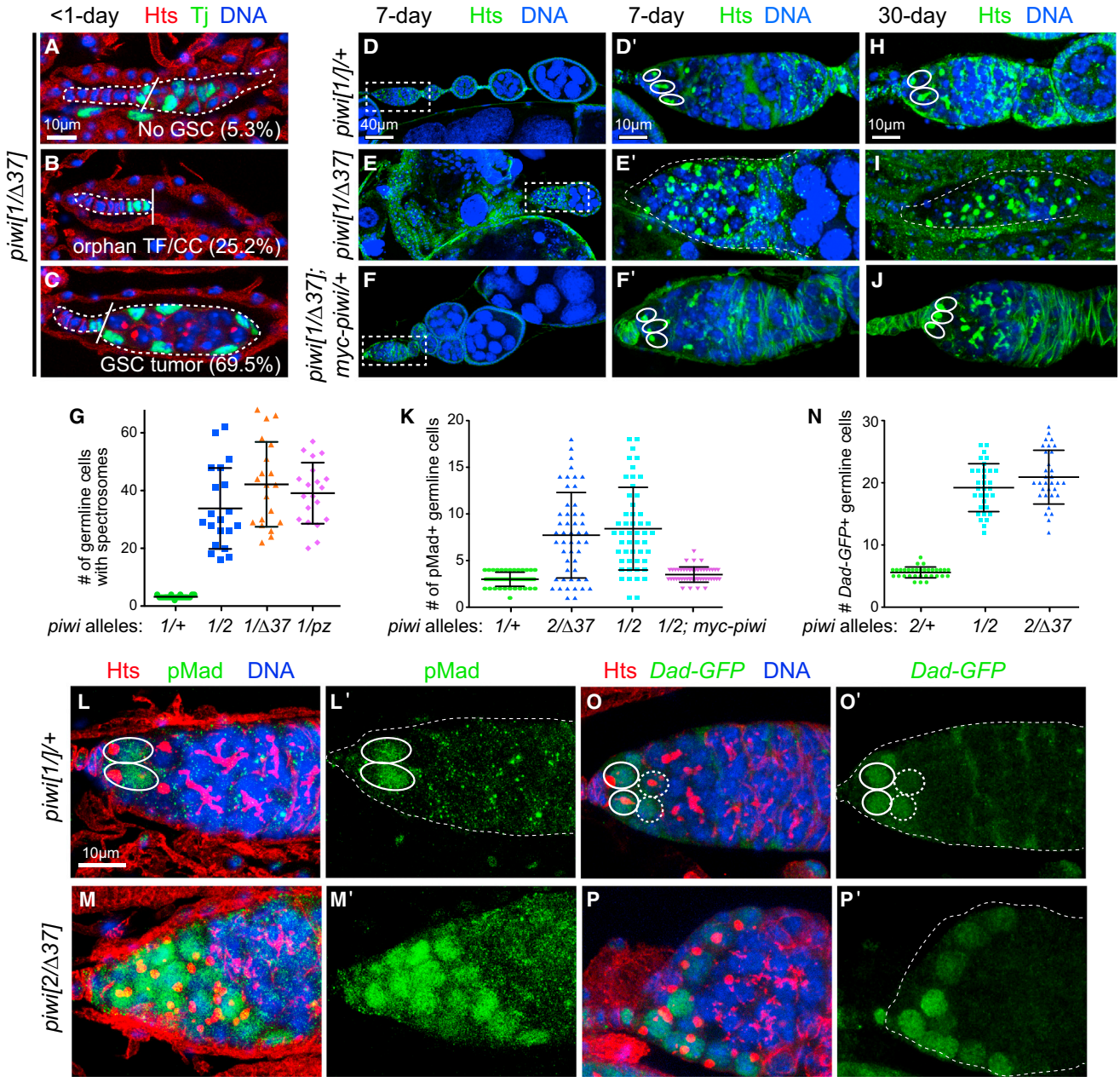

Figure 1. *piwi* Mutants Exhibit Predominant Germline Stem Cell-like Tumors and Elevated Dpp Signaling

(A–C) Partial or whole germaria in <1-day-old *piwi[1/Δ37]* ovaries stained for Hts (red), Tj (green), and DNA (blue) and quantified as noted.
(A) Example of germarium lacking germline stem cells (GSCs).
(B) Partial germarium consisting of "orphan" terminal filaments (TFs) and cap cells (CCs).
(C) Example of germarium displaying a GSC tumor.
(D–F) Egg chambers are abbreviated in *piwi* mutants and exhibit GSC-like tumors; these phenotypes are rescued by *myc-piwi*. Select germarium regions are boxed and enlarged in (D′)–(F′), highlighting approximately three GSC cells with ball spectrosomes (marked with white circles) in *piwi* heterozygous (D′) and rescued *piwi* mutants (F′) but GSC-like tumors filling the germaria of *piwi* mutants (E′).
(G) Quantification of spectrosome-containing cells in 7-day-old *piwi* heterozygous and mutant ovarioles.
(H–J) 30-day-old germaria of the respective genotypes resemble their 7-day-old counterparts.
(K) Quantification of germline cells expressing the GSC marker pMad in different *piwi* genotypes.
(L and M) Representative staining of pMad in *piwi* heterozygous (L) and mutant (M) germaria.
(N) Quantification of germline cells expressing the GSC/cystoblast marker *Dad-GFP* in different *piwi* genotypes.
(O and P) Representative *Dad-GFP* expression in *piwi* heterozygous (O) and mutant (P) germaria.
Error bars in (G), (K), and (N) represent mean with SD. See also Figure S1.

Nevertheless, in the remaining germaria, we still observed large numbers of spectrosome-containing cells in 1-month-old *piwi* mutants, and these phenotypes could be fully rescued (Figures 1H–1J and S1L). Altogether, we find clear evidence for GSC-like tumors that persist throughout *piwi* mutant adult life. Since we were currently unable to

distinguish whether orphan TF/CC structures are attributable to GSC loss or to defective GSC recruitment, we focused our subsequent analyses and quantifications on ovarioles bearing intact germaria.

Ovarian GSCs are maintained by the activity of nuclear pMad. In newly born and 2- to 3-day-old ovaries (n = 50 for all genotypes), control *piwi* heterozygous ovaries contained 3.0 ± 0.1 pMad+ cells (Figure 1K). In contrast, *piwi[1/2]* exhibited 8.4 ± 0.6 and *piwi[2/Δ37]* exhibited 7.7 ± 0.7 pMad+ germline cells; this defect was fully rescued by *myc-piwi* to 3.5 ± 0.1 pMad+ cells (Figure 1K). Representative stains of wild-type and *piwi* germaria in Figures 1L and 1M highlight that the ectopic pMad+ cells exhibit ball-shaped spectrosomes instead of branched fusomes typical of multicell cystocytes, attesting to their undifferentiated status. A direct downstream transcriptional target of pMad is *Daughters against dpp* (*Dad*), which can be monitored by expression of *Dad-GFP*. Indeed, we observed ectopic *Dad-GFP* expression in different *piwi* mutants (19.2 ± 0.7 in *piwi[1/2]* and 20.9 ± 0.8 in *piwi[2/Δ37]*; n = 32; Figure 1N); representative germaria are shown in Figures 1O and 1P. Overall, *piwi* mutations appear to elevate levels of Dpp signaling in the majority of undifferentiated germline cells, thus keeping them in GSC-like and/or cystoblast-like stages.

A critical function of Dpp signaling in GSCs is to repress the transcription of *bam*. We observed drastic reduction of Bam+ germline cells (Figures 2A–2C), from 92% of control germaria (Figure 2A, white outlined regions; n = 149) to 4.1% in *piwi[1/Δ37]* (n = 171) and 9.2% in *piwi[1/2]* mutants (Figures 2B and 2C; n = 185). This was a transcriptional effect, since a *bam-GFP* reporter that is normally activated in late cystoblasts and other differentiating germline cells (Figure 2D) was largely silent in *piwi[2/Δ37]* (n = 57) and *piwi[1/2]* (n = 48) mutants (Figures 2E and 2F, white outlined areas). To assess whether loss of Bam is causal for *piwi* GSC-like tumors, we introduced a *hs-bam* transgene into *piwi* mutants. While heat shock alone was unable to rescue fusome morphology in *piwi* mutants (Figures 2G and 2H), "branched" fusome morphology was regained in *hs-bam*, *piwi* mutants 18 hr after heat shock (n = 46 germaria; Figures 2I and 2J, dashed circles). Consistent with this, induction of hs-Bam also reduced ectopic pMad staining in *piwi* mutants, even eliminating endogenous staining (Figures 2K and 2L). We conclude that the lack of *bam* expression in *piwi* germline cells is a major cause of their GSC-like tumors.

To gain insight into the spatial requirement for Piwi function, we used several cell-type-specific Gal4 lines for rescue experiments. Consistent with previous reports that Piwi is required primarily in the soma to maintain overt ovarian morphology [4], expression of Piwi using the germline driver *nanos-Gal4* did not rescue *piwi* tumors (Figures S3A and S3B). *hh-Gal4* is active in the somatic niche, i.e., in TFs and CCs (Figure S2A). Reporter analysis showed that *hh-Gal4* activity is not maintained in all *piwi* mutant germaria (Figures S2B–S2D); therefore, we scored only germaria with demonstrated Piwi expression in CCs (Figures S2E and S2F). Surprisingly, expression of Piwi using *hh-Gal4* did not rescue *piwi* tumors (Figure S3C), suggesting that Piwi is not required in the cells that are believed to be the normal source of niche Dpp. The *tj-Gal4* driver is active in CCs, ECs, and other follicle cells [12] (except stalk cells; Figures S2G and S2H). Interestingly, expression of Piwi in Tj+ cells completely suppressed *piwi* germline tumors and restored egg production (Figure S3D), although these eggs could not hatch, likely due to germline Piwi function [13, 14]. The *c587-Gal4* driver has similar specificity to *tj-Gal4* in germarial areas, except that it is not active in CCs (Figures S2J and S2K). We also observed full rescue of GSC dynamics and ovary morphology using *c587-Gal4* to activate Piwi (Figures 3A and S3E), implying that Piwi is predominantly required in ECs.

In reciprocal tests, we asked whether cell-type-restricted expression of *UAS-piwi-RNAi* could induce germline tumors. As with the rescue experiments involving *hh-Gal4*, we only scored knockdown germaria for which we confirmed Piwi loss in CCs (Figures S2M and S2N). We failed to find ectopic GSC-like cells upon induction of constitutive *piwi* knockdown using *hh-Gal4* (4.6 ± 0.3, n = 30, p = 0.26) even when performing the experiment in a *piwi* heterozygous background to sensitize the knockdown (Figure S3F). On the other hand, knockdown of *piwi* using either *c587-Gal4* (12.8 ± 7.4, n = 30, p < 0.0001; Figure 3E) or *tj-Gal4* (13.1 ± 6.2, n = 30, p < 0.0001; Figure S4E) induced large numbers of ectopic GSC-like cells (Figures S3G and S3H).

Because Dpp is not normally considered to influence GSC dynamics via ECs, we sought evidence that Dpp is required for *piwi* mutant GSC tumors via ECs. We observed GSC loss in 26% of *c587-Gal4*-driven *dpp-RNAi* germaria, which potentially suggested interference of Dpp signaling in niche cells during development (data not shown). To bypass potential developmental effects, we used the *tub-Gal80[ts]* system to temporally control *dpp* knockdown. A regimen in which Dpp was silenced for 4 days by shifting adult flies to 29°C rescued GSC-like tumors and promoted germline differentiation in 64.7% *piwi* mutant germaria (Figures 3B and S3I; n = 68). This provided compelling evidence that continuous upregulation of Dpp signaling in ECs is a primary cause of *piwi* germline tumors in adult stages.

We subsequently investigated temporally controlled manipulations of Piwi. Control *tub-Gal80[ts];c587-Gal4>UAS-piwi-RNAi* females raised at the permissive temperature showed normal Piwi staining and ovary morphology (Figures 3C and S4A). In contrast, siblings shifted to the restrictive temperature showed specific loss of Piwi protein in somatic cells (Figures 3D and S4B). This was accompanied by derepression of Gypsy transposons, as evidenced by accumulation of Gypsy envelope protein (Figure 3C″ versus 3D″). Surprisingly, this adult-specific regimen for Piwi depletion did not significantly induce ectopic spectrosome-containing germline cells (Figure 3E). We therefore assayed other developmental stages. We observed that *c587-Gal4*-mediated knockdown of *piwi* specifically in pupal stages caused a mild increase in GSC-like cells but that restricted knockdown in wandering third-instar larvae led to a substantial *piwi* phenocopy (Figure 3E). We obtained identical results by depleting *piwi* using temporally controlled activity of *tj-Gal4* (Figures S4C–S4E). We complemented these tests by resupplying Piwi in *piwi* mutants at various developmental stages using *tub-Gal80[ts]* and *c587-Gal4*. We obtained substantial rescue when Piwi was induced transiently in wandering third-instar larvae, but not at pupal or adult stages (Figure 3F). These experiments established that Piwi is critically required during wandering third-instar larval stages.

During niche formation in larval gonads, intermingled cells (ICs) are essential for the differentiation of primordial germline cells (PGCs, the progenitor cells of adult germline stem cells) residing outside of niches. We confirmed that Tj-positive ICs

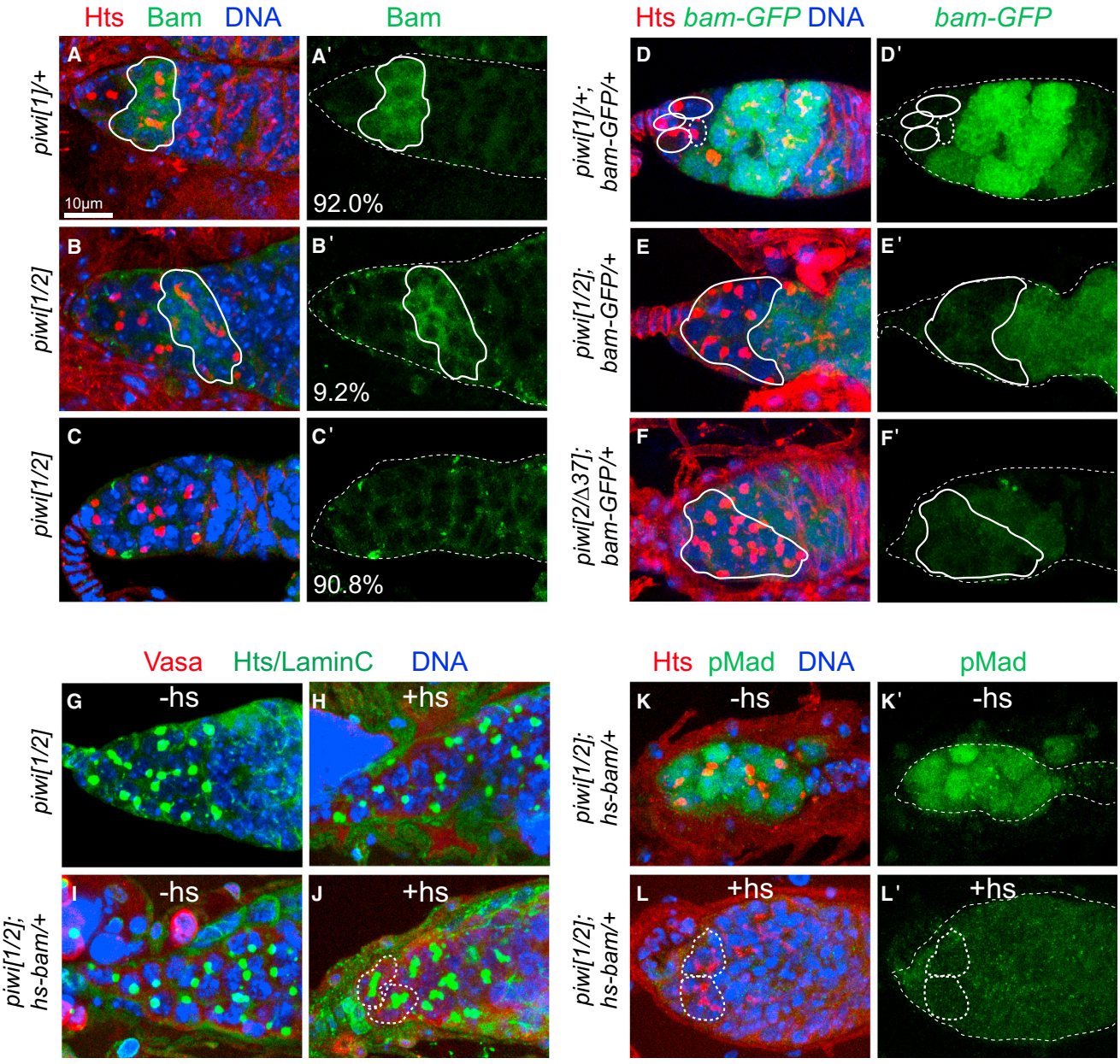

Figure 2. Failure of GSC Differentiation in *piwi* Mutants Is Due to Loss of Bam Expression

(A) *piwi[1]/+* heterozygote illustrates normal accumulation of Bam protein in differentiating cystocytes (white lines).

(B and C) Almost 10% of *piwi[1/2]* mutant ovaries initiate Bam expression (B, white lines), but the vast majority fail to express Bam (C). Bam staining is shown separately in (A′)–(C′).

(D) *piwi[1]/+* heterozygote illustrates a normal pattern of *bam-GFP* expression that begins in differentiating cystocytes—germline cells other than GSCs (white circles) and cystoblasts (dashed circle).

(E and F) *piwi[1/2]* (E) and *piwi[2/Δ37]* (F) mutants fail to activate *bam-GFP* in those ectopic GSC-like cells (white line areas). Bam-GFP staining is shown separately in (D′)–(F′).

(G–J) Rescue of *piwi* mutants by ectopic Bam.

(G and H) *piwi* mutant exhibits GSC-like tumors (G), and these persist following heat shock (H).

(I and J) In *piwi* mutants bearing *hs-bam*, spectrosome morphology changes from ball-shaped before heat shock (I) to branched after a heat-shock treatment (J, areas inside dashed lines).

(K) *piwi[1/2];hs-bam/+* germarium exhibits many ectopic pMad+ cells with ball-shaped spectrosomes without a heat-shock treatment.

(L) Following heat shock, *piwi[1/2];hs-bam/+* germarium exhibits pMad-negative cells with branched fusomes (dashed lines) in the niche. pMad staining is shown separately in (K′)–(L′).

in *piwi* gonads are not mixed with germline cells as in the control [15] but instead remain on the periphery of the germline cell mass (Figures 4A and 4B). Concomitant with this was the failure to upregulate *bam-GFP* in *piwi* mutant larval gonads (Figures 4C and 4D), similar to its behavior during adult stages (Figures 2A–2F). Moreover, ECs derived from IC lineages exhibited defective morphology, since membrane extensions labeled by *c587-Gal4>UAS-CD8-GFP* were clearly reduced in

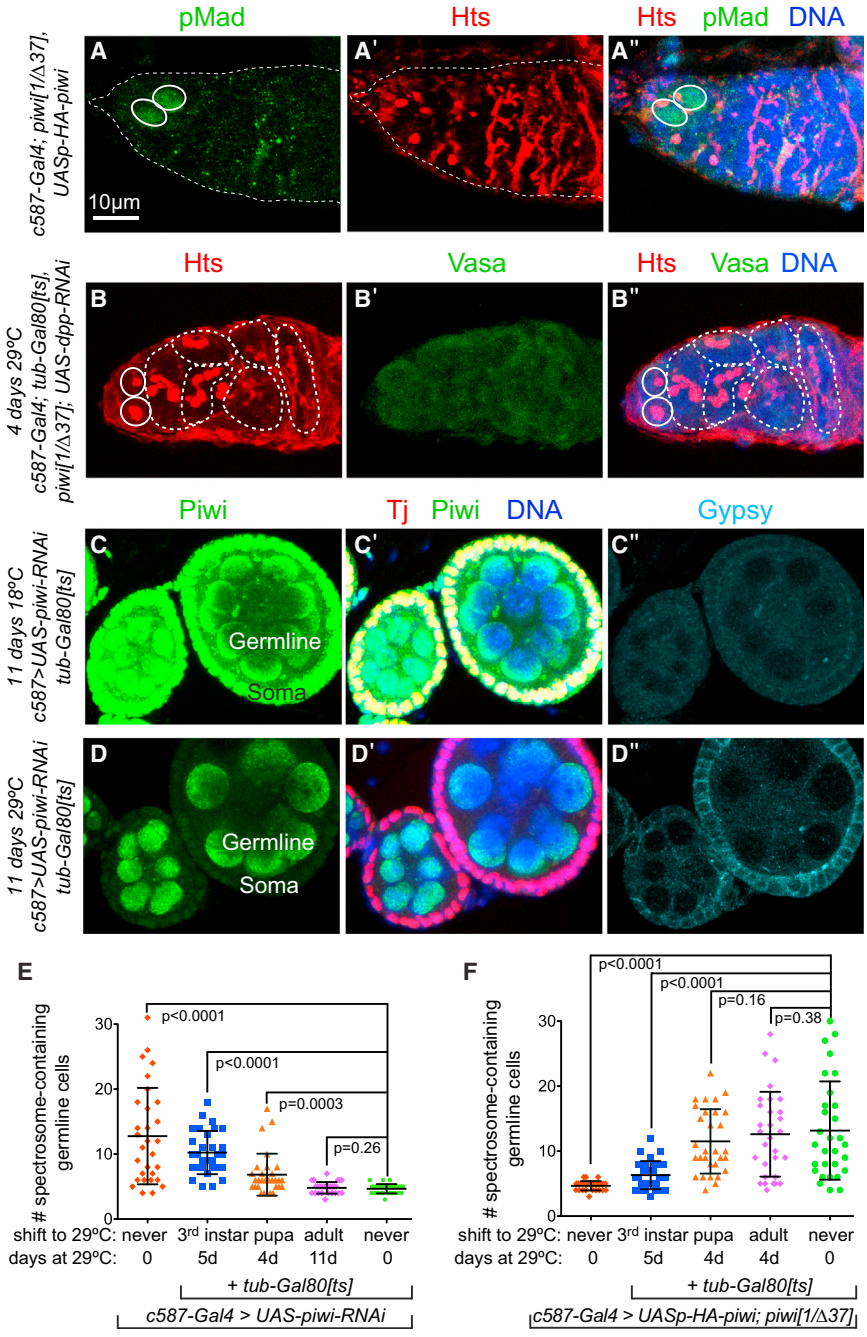

Figure 3. Spatial and Temporal Function of Dpp Signaling and Piwi for Normal GSC Differentiation

(A) *piwi* GSC-like tumors are rescued by expression of *piwi* using *c587-Gal4*, which is active in escort cells (ECs) and follicle cells. White circles highlight two pMad+ GSCs, characteristic of wild-type.

(B) Adult-specific rescue of *piwi* mutant germaria by temporally controlled induction of *dpp-RNAi*. Inclusion of *tub-Gal480[ts]* in the background restricts Gal4 activity until shifting to the restrictive temperature (29°C) in adult stages. Ovaries of this genotype remain small due to the developmental defect, but examination of their germaria indicates rescue of normal GSC numbers (white circles) and the presence of differentiating cysts (dashed lines).

(C and D) Validation of cell-type- and adult-specific knockdown of Piwi. Females of the genotype *c587-Gal4, UAS-piwi-RNAi;tub-Gal80[ts]* were raised at 18°C until eclosion and then maintained at 18°C (C) or shifted to 29°C (D) for 11 days of adult life prior to immunostaining for Piwi, Tj, and Gypsy-envelope proteins. Late-stage egg chambers exhibit normal Piwi and Tj accumulation and lack Gypsy reactivity in controls (C–C″), whereas animals shifted to the restrictive temperature specifically lack Piwi in somatic cells and exhibit derepression of Gypsy (D–D″).

(E) Knockdown of *piwi* using cell-type and temporal control demonstrates that GSC-like tumors can be generated by specifically depleting Piwi using *c587-Gal4* during late larval stages.

(F) Expression of Piwi with cell-type and temporal control in *piwi* mutants shows that GSC-like tumors can only be substantially rescued when providing Piwi at the third instar using *c587-Gal4*. See also Figures S2–S4. Error bars in (E) and (F) represent mean with SD.

piwi mutants (Figures 4E and 4F); EC numbers were also reduced (Figure 4G). Altogether, the temporal requirement for Piwi in larval gonad development suggested that its absence leads to inappropriately sustained Dpp signaling in ICs, which in turn impedes the developmental switch of PGC differentiation. This appears to be mediated at least partly by defective differentiation of IC-derived lineages, including of ECs whose cellular extensions are important for ovarian GSC differentiation [16].

In summary, we analyzed multiple heteroallelic backgrounds, genetic rescues, and temporally controlled manipulations to show that the predominant effect of *piwi* loss is elevation of Dpp signaling leading to GSC tumors. We demonstrate this using multiple readouts of GSC fate and Dpp pathway status and further show that *piwi* mutant tumors were partially rescued by somatic knockdown of *dpp* or re-expression of Bam in the germline. Curiously, while our spatially and temporally controlled manipulations demonstrate that *piwi* GSC tumors are actively sustained in the adult by excess Dpp signaling, the direct requirement for Piwi for normal germline formation is largely restricted to larval stages. Tj-positive ICs do not mix with PGCs in late-third-instar *piwi* gonads, which coincides with the failure of PGCs to express the differentiation factor Bam. We propose that *piwi* adult ovarian phenotypes stem from a failure of ICs to downregulate *dpp* and/or differentiate into ECs properly.

Further investigation is needed to establish the molecular mechanism by which Dpp signaling is deregulated. In principle, this might be a direct consequence of defective Piwi-interacting RNA (piRNA)-mediated silencing or an indirect cause of defective EC differentiation [16]; these possibilities are not exclusive. Finally, while the majority of *piwi* ovarioles exhibit GSC tumors, we note a substantial population of aberrant structures consisting of orphan TF/CC clusters that are not associated with germline cells (Figure 1). These appear distinct from the rare class of intact ovarioles exhibiting overt

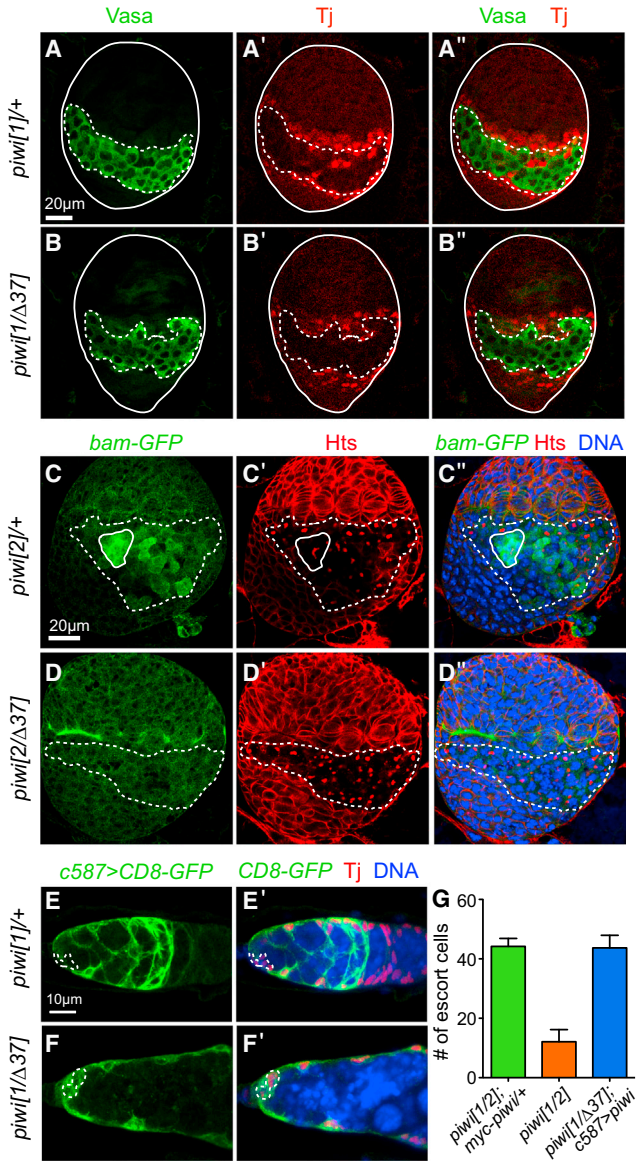

Figure 4. Somatic Defects in *piwi* Mutant Larval Gonads Are Associated with Defective Escort Cell Morphology

(A–D) Immunostaining of wandering third-instar larval gonads; dashed lines delineate areas containing primordial germline cells.

(A and B) Intermingling of somatic cells (marked by Tj) and germline cells (marked by Vasa) occurs in *piwi* heterozygotes (A), but not in *piwi[1/Δ37]* mutants (B).

(C and D) Upregulation of the germline differentiation marker *bam-GFP* occurs in *piwi* heterozygotes (C, circled), but not in *piwi[2/Δ37] mutants* (D), and only the former exhibits cells with branched fusome structures (C′, circled).

(E and F) Escort cells in adult *piwi* heterozygous germaria exhibit characteristic cellular extensions (E), but these are largely missing in *piwi[1/Δ37]* mutants (F). Note that CCs (dashed line areas) are labeled in *piwi* mutants (F), but not in controls (E).

(G) Loss of *piwi* also reduces the number of ECs. Error bar in (G) represents mean with SD.

GSC loss and raise the question of whether *piwi* is also required for effective recruitment of GSCs during niche formation. Additional studies are needed to clarify the developmental impact of Piwi loss during different aspects of ovary development.

Experimental Procedures

Drosophila Strains

Fly crosses were performed at 25°C unless otherwise stated. We analyzed the *piwi* alleles and transgenes *piwi[1]*, *piwi[2]*, *piwi[pz06843]* (designated as *piwi[pz]*), *Df(2L)[Δ37]* (a small deletion that affects *scar* and *piwi* [11], hereafter designated as *piwi[Δ37]*), *myc-piwi*, and *UAS-piwi-RNAi* (Bloomington Stock Center [BSC] #33724). For cell-type-specific expression and rescue experiments, we used *UASp-HA-piwi* driven with *c587-Gal4* (from Ting Xie), *tj-Gal4* (from Dorothea Godt), *act5C-Gal4* (from Ruth Lehmann), *hh-Gal4*, *nos-Gal4*, and *tub-Gal80[ts]* (from BSC). Analysis of Dpp signaling utilized *Dad-GFP* [17] and *UAS-dpp-RNAi* (BSC #31172 and #33767); nearly all *tj-Gal4>UAS-dpp-RNAi* ovaries lacked GSCs, validating their potency (data not shown). Analysis of Bam function utilized *bam-GFP* and *hs-bam*. To express Bam in *piwi* mutants, we subjected *piwi[1], hs-bam/piwi[2]* flies to two 1 hr heat shocks at 37°C separated by a 2 hr recovery period at 25°C; ovaries were analyzed 18 hr after the first heat shock. For crosses involving *tub-Gal80[ts]*, flies were first cultured at 18°C to desirable developmental stages and then upshifted to 29°C to activate *UAS* transgenes in specific cells driven by individual *Gal4* lines.

Immunostaining

Ovaries from adult flies or larval gonads were dissected and fixed in PBS containing 4% formaldehyde. Mouse anti-Hts (1:10, Developmental Studies Hybridoma Bank [DSHB]), rabbit anti-GFP (1:2,500, Clontech), rat anti-Vasa (1:50, DSHB), rabbit anti-pMad (1:2,000, gift from Ed Laufer and Tom Jessel), guinea pig anti-Tj (1:10,000, gift from Dorothea Godt), rabbit anti-caspase 3 (1:200, Cell Signaling), mouse anti-Bam (1:10, DSHB), mouse anti-Piwi (1:10, gift from Haruhiko and Mikiko Siomi), and rabbit anti-Gypsy Env (gift from Alain Pelisson) were used. Alexa Fluor 488, 568, and 647 secondary antibodies were from Molecular Probes and were used at 1:500. An Apoptosis Detection Kit (Millipore) was used for TUNEL assay. Images were captured with a Leica TCS confocal microscope.

Supplemental Information

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.021.

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